LASER-COMPATIBLE NIR-MARKER DYES

The invention is directed to so-called laser-compatible NIR marker dyes based on polymethines for use in optical, especially fluorescence-optical, methods for determination and detection. Typical method applications are based on the reaction of dye-labeled antigens, antibodies or DNA segments with the respective complementary species. Possible uses are, for example, in medicine and pharmacology, biological and material sciences, environmental control and for detection of naturally occurring organic and inorganic microprobes, and so forth.

Polymethines have long been known as NIR markers and are distinguished by intensive absorption maxima which can easily be shifted in the NIR region (Fabian, J.; Nakazumi, H.; Matsuoka, M.: *Chem.-Rev.* 1992, 92, 1197). With a suitable substituent pattern and π-electron system, they fluoresce with sufficient quantum yield also in the NIR region. Accordingly, these compounds are commonly applied in different areas of technology as sensitizers in AgX materials, as laser dyes, and quantum counters, as indicator dyes in sensor engineering and, also importantly, as biomarkers ("Near-infrared Dyes for High Technology Applications", edited by Daehne, S., Resch-Genger, U.; Wolfbeis, O.-S., Kluwer, Academic Publishers - Dordrecht/Boston/London 1998). The number of polymethines used as biomarkers is limited. In this connection, only the following have achieved widespread commercial application heretofore: trimethine Cy3 derived from astraphloxine (DE 415 534) or the vinylogous pentamethine Cy5 and the double-vinylogous heptamethine Cy7 with absorption maxima at around 550 nm, 650 nm and 750 nm (US-PS 5,627,027). Further, the polysulfonated trimethine Cy3.5 and pentamethine Cy5.5 derived from the commercial heptamethine "Indocyanine Green" and "Cardio Green" are available (US-PS 5,569,766). Heptamethines which are aliphatically bridged in the polymethine chain have been developed by Patonay (US-PS 5,800,995). The terminal heteroaromatics deriving from indene (Fischer's base) and heteroindene are characteristic of all commercial biomarkers. When methyl-substituted cycloimmonium salts of this type are used as terminal polymethine building blocks, it is necessary to arrange at least five successive sp^2-hybridized carbon atoms (pentamethine) between the heterocycles in order to generate absorption maxima at the boundary of the NIR region.

A substantial disadvantage of the NIR polymethines in technical use as biomarkers consists in that lengthening of the polymethine chain increases the possibility of nucleophilic or electrophilic attack on the chain resulting in destruction of the π-system. Apart from the unsatisfactory thermal and photochemical stability, another substantial defect of polymethines consists in that they have no other absorption bands in the visible spectral region aside from their intensive absorption maxima and cannot be directly excited in this spectrum, particularly by argon lasers with an emission wavelength of λ_{em} = 488 nm or He-Ne lasers with λ_{em} = 633 nm or corresponding laser diodes from λ_{em} = 670 nm. In particular, biomarkers which are suitable for multiple color fluorescence assays can be excited only by discrete light sources (such as those mentioned above) predetermined by the π-system of the polymethine. In order to make such applications possible in spite of this (when using multiple color fluorescence assays it is necessary to excite different biomarkers, for example, with one of these excitation light sources, with clearly distinguishable emission maxima), the excitation of Cy5 is carried out by an argon laser, for example, in that an emission is caused by the excitation of light at the boundary of the NIR region by means of energy transfer by excitation of fluorescein → rhodamine → Texas Red → Cy5 (US-PS 5,800,996). Other possibilities for excitation of Cy5, for example, by means of an argon laser, include generation of microparticles from intrinsic fluorophores (phycobiliproteins) and extrinsic Cy5 which permit the Cy5 derivatives absorbing at 650 nm to be excited by energy cascades (Szöllösi, J.; Damjanovich, S.; Matyus, L.: *Cytometry* 1998, 34, 159).

Gupta (US-PS 5,783,673) describes dye conjugates which were prepared by the reaction of phycobiliprotein with activated fluorescein, Texas Red or Cy5-dyes (phycobiliprotein/amine-reactive dye - PARD). The dye conjugates obtained in this way show additional absorption bands in the visible spectral region which can be utilized for excitation. These probes have the disadvantages of high molecular mass, uneconomical preparation and low stability of the marker dyes.

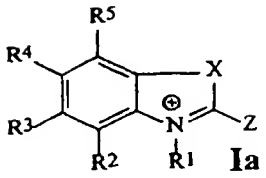
Another example for the excitation of pentamethines which are not absorbent at 488 nm is given by Glazer (US-PS 5,760,201). In addition, a strong affinity to DNA is achieved (specific ion bonding) by covalent linking with a monomethine absorbing in the desired region by way of a plurality of ammonium-

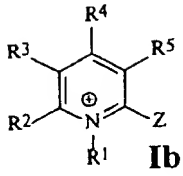
containing optimized alkyl spacers. A correspondingly complicated process for excitation is also unavoidable in this case. Further disadvantages of these marker dyes are insufficient photostability and storage stability, costly synthesis and purification steps, low absorption coefficients and unsatisfactory fluorescence quantum yields, as well as unwanted changes in optical characteristics in the presence of or after bonding to proteins or nucleic acid oligomers.

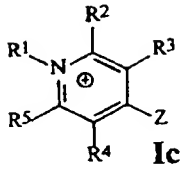
Object and Summary of the Invention

It is the object of the invention to provide polymethine-based NIR marker dyes with high photostability and storage stability as well as high fluorescence yields which can be excited to fluorescence in the simplest possible manner by laser radiation in the visible or near-IR spectral regions, especially by light from an argon laser, helium-neon laser or diode laser.

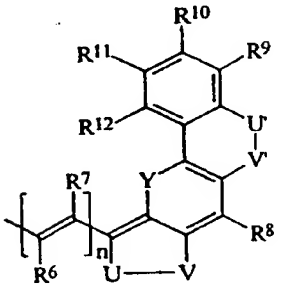
The invention uses polymethine-based marker dyes which contain substituted derivatives of benzooxazole, benzothiazole, 2,3,3-trimethylindolenine, 2,3,3-trimethyl-4,5-benzo-3*H*-indolenine, 2- and 4-picoline, lepidine, chinaldine and 9-methylacridine of the general formula Ia or Ib or Ic


Ia

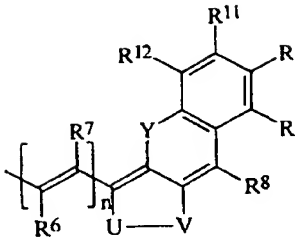

Ib


Ic

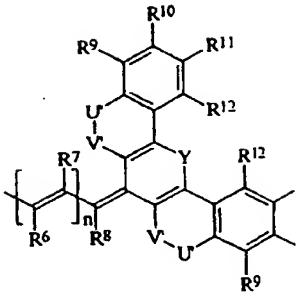
where Z is



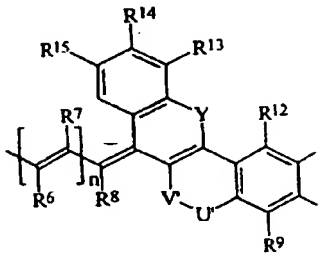
or



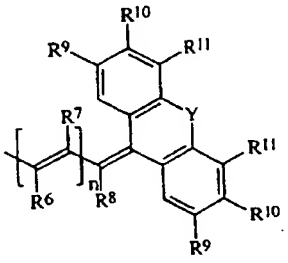
or



or



or



wherein

- X or Y is an element from the group comprising O, S, Se or the structural element N-alkyl or $C(alkyl)_2$,
- n represents the numerical value 1, 2 or 3,

- R^1 - R^{15} are identical or different and can be hydrogen, one or more alkyl- or aryl-, heteroaryl- or heterocycloaliphatic groups, a hydroxy or alkoxy group, an alkyl-substituted or cyclic amine function and/or two *ortho* groups, e.g., R^2 and R^3, together can form another aromatic ring,
- at least one of the substituents R^1 - R^{15} can be an ionizable or ionized substituent such as SO_3^-, PO_3^{2-}, COO^- or NR_3^+ which determines the hydrophilic characteristics of these dyes,
- at least one of the substituents R^1 - R^{15} can represent a reactive group which enables a covalent linking of the dye with the carrier molecules mentioned above, and
- U-V or U'-V' are identical or different and can comprise hydrogen, a saturated aliphatic, heteroaliphatic or a lactone or thiolactone grouping.

Special embodiment forms for the marker dyes are given in subclaims 2 - 10.

These substituted indole, heteroindole, pyridine, chinoline or acridine derivatives of the general formula Ia or Ib or Ic can be used as dyes for optical labeling of organic or inorganic microparticles, e.g., proteins, nucleic acids, DNA, biological cells, lipids, drugs or organic or inorganic polymeric carrier materials. The labeling of the particles can be carried out by forming ionic interactions between the markers of the general formulas Ia or Ib or Ic and the materials to be labeled.

The functional groups of these markers which are activated relative to the nucleophiles are capable of covalent coupling to an OH-, NH_2- or SH-function. This results in a system for qualitative or quantitative determination of organic and inorganic materials such as said proteins, nucleic acids, DNA, biological cells, lipids, drugs or organic or inorganic polymers.

Brief Description of the Drawings

This coupling reaction can be carried out in aqueous or predominantly aqueous solution, preferably at room temperature. A conjugate with fluorescent characteristics is formed in this way.

The compounds of the general formulas Ia or Ib or Ic and systems derived therefrom can be used in optical, especially fluorescence-optical, qualitative and quantitative determination processes for diagnosing cell characteristics, in biosensors (point-of-care measurements), genome research, and in miniaturization

technologies. Typical applications are in cytometry and cell sorting, fluorescence correlation spectroscopy (FCS), ultra-high throughput screening (UHTS), multicolor fluorescence in-situ hybridization (FISH) and microarrays (genchips).

Through the preparation of nonsymmetric polymethines which, on the one hand, as terminal function, have an easily derivable heterocycle of the pyridine, chinoline, indole, heteroindole and acridine derivative types and, on the other hand, have a novel 6-ring heterocycle, the following advantages are achieved in particular.

Even trimethines absorb in the spectral region of greater than 650 nm and show a substantially improved photochemical and thermal stability in comparison to the previously known polymethines with absorption maxima greater than 650 nm (pentamethine and heptamethine).

Molecular engineering makes it possible to control the position and intensity of the absorption maxima and emission maxima in any desired manner and to adapt the emission wavelengths of different excitation lasers, especially NIR laser diodes.

Due to the selection of suitable terminal heterocycles, the dyes according to the invention show additional absorption maxima in the visible and NIR spectral region which can be utilized for excitation, for example, with an argon laser. These dyes are particularly suited to application in multiple color fluorescence assays.

The marker dyes can be produced by means of relatively simple syntheses which are carried out in two steps and by which a large number of variously functionalized dyes, e.g., with respect to total charge of the dye and the quantity, specificity and reactivity of the activated groups used for immobilization, can be provided for specific applications.

The invention will be described more fully in the following with reference to embodiment examples shown in the drawing.

Fig. 1 shows syntheses according to embodiment examples 1 and 2;

Fig. 2 shows a synthesis according to embodiment example 3;

Fig. 3 shows syntheses according to embodiment examples 4 to 6;

Fig. 4 shows syntheses according to embodiment examples 7 and 8;

Fig. 5 shows absorption spectrum of C 1601;

Fig. 6 shows emission spectrum of C 1601 (free, bonded, 670-nm diode laser);

Fig. 7 shows syntheses according to embodiment examples 11 and 12;

Fig. 8 shows syntheses according to embodiment examples 13 and 14;

Fig. 9 shows absorption spectrum of C 1591 NHS ester;

Fig. 10 shows emission spectrum of C 1591 (free, bonded, 670-nm diode laser);

Fig. 11 shows emission spectrum of C 1591 (free, bonded, 488-nm Ar-ion laser); and

Fig. 12 shows syntheses according to embodiment examples 19 and 20.

Detailed Description of the Invention

General directions for preparing 3,1-bridged 2-(2-ethoxyethenyl)-7-diethylamino-benzo[b]pyrylium perchlorates C 1595 and L 107, see Fig. 1:

0.01 mol of a 2-methylene-7-diethylamine-benzo[b]pyrylium perchlorate of formula 1a or 1b is dissolved in 40 ml acetic anhydride and briefly heated with 2.0 g triethoxymethane. The precipitate occurring after approximately one hour is sucked off and recrystallized from glacial acetic acid.

1: *6-Diethylamino-4-ethoxymethylene-1,2,3,4-tetrahydro-<dibenzo[b;e]pyrylium> perchlorate* C 1595: Yield: 3.58 g (87%); melting point:

178°C. $^{-1}$H NMR ($CDCl_3$): 1.29 (t, *J* = 7.1 Hz, 6H), 1.42 (t, *J* = 7.1 Hz, 3H), 1.78-1.82 (m, 2H), 2.54 (t, *J* = 6.0 Hz, 2H), 2.75 (t, *J* = 5.8 Hz, 2H), 3.59 (q, *J* = 7.1 Hz, 4H), 4.53 (q, *J* = 7.1 Hz, 2H), 6.93 (dd, *J* = 2.3, *J* = 9.3 Hz, 1H), 7.32 (d, *J* = 2.0 Hz, 1H), 7.50 (d, *J* = 9.3 Hz, 1H), 7.84 (s, 1H), 8.52 (s, 1H). $^{-13}$C NMR ($CDCl_3$): 12.5, 15.6, 20.2, 21.8, 27.8, 45.8, 73.1, 97.1, 108.3, 115.4, 115.8, 120.3, 130.6, 145.6, 154.8, 157.8, 163.0, 167.9. $^{-}C_{20}H_{26}ClNO_6$ (411.88): calculated C 58.32, H 6.36, Cl 8.61, N 3.40, actual C 57.75, H 6.58, Cl 8.43, N 3.46.

2: *3-Diethylamino-6-ethoxymethylene-7,8,9,10-tetrahydro-6H-<5-oxonia-cyclohepta[b]-naphthalene> perchlorate* L 107: Yield: 3.96 g (93%); melting point: 158-60°C. $^{-1}$H NMR ($CDCl_3$): 1.27 (t, *J* = 7.1 Hz, 6H), 1.39 (t, *J* = 7.1 Hz, 3H), 1.75-1.77 (m, 2H), 1.85-1.87 (m, 2H), 2.58-2.61 (m, 2H), 2.79-2.83 (m, 2H), 3.58 (q, *J* = 7.1 Hz, 4H), 4.56 (q, *J* = 7.1 Hz, 2H), 6.99 (dd, *J* = 2.4, *J* = 9.3 Hz, 1H), 7.16 (d, *J* = 2.0 Hz, 1H), 7.60 (d, *J* = 9.3 Hz, 1H), 8.00 (s, 1H), 8.18 (s, 1H). $^{-13}$C NMR ($CDCl_3$): 12.5, 15.5, 21.1, 23.8, 25.1, 29.2, 45.8, 72.4, 96.3, 113.2, 116.1, 116.3, 124.2, 130.8, 149.0, 155.0, 157.9, 162.8, 171.0. $^{-}C_{21}H_{28}ClNO_6$ (425.91): calculated C 59.22, H 6.63, Cl 8.32, N 3.29, actual C 58.76, H 6.39, Cl 8.75, N 3.34.

3: *3-Diethylamino-6-[3-(N-acetylanilino)-prop-2-ylidene]-7,8,9,10-tetrahydro-6H-<5-oxonia-cyclohepta[b]naphthalene> percholate C 1590*, see Fig. 2: 2.13 g (0.005 mol) of 2-methylene-7-diethylamine-benzo[b]pyrylium perchlorate of formula 1b are dissolved in 40 ml acetic anhydride and briefly heated with 1.29 g (0.005 mol) (3-anilinopropenylidene)-phenyl-ammonium chloride. The precipitate occurring after approximately one hour is sucked off, washed with ether and recrystallized from glacial acetic acid: Yield: 2.00 g (74%); melting point: 216-20°C. $^{-1}$H NMR (CD_3NO_2): 1.34 (t, *J* = 7.1 Hz, 6H), 1.64-1.69 (m, 2H), 1.82-1.87 (m, 2H), 2.00 (s, 3H), 2.49 (t, *J* = 6.0 Hz, 2H), 2.89 (t, *J* = 6.0 Hz, 2H), 3.72 (q, *J* = 7.1 Hz, 4H), 5.61 (dd, *J* = 11.8 Hz, *J* = 13.5 Hz, 1H), 7.04 (d, *J* = 2.4 Hz, 1H), 7.32 (dd, *J* = 2.4 Hz, *J* = 9.4 Hz, 1H), 7.36-7.39 (m, 2H), 7.54-7.65 (m, 4H), 8.21 (s, 1H), 8.27 (d, *J* = 13.5 Hz, 1H), $^{-13}$C NMR (CD_3NO_2): 12.8, 23.5, 25.2, 25.8, 25.9, 30.4, 47.2, 96.2, 109.9, 119.0, 119.3, 127.4, 129.9, 130.6, 130.7, 131.7, 132.0, 132.5, 140.1, 142.2, 151.3, 157.2, 160.1, 169.7, 171.2. $^{-}C_{29}H_{33}ClN_2O_6$ (541.04): calculated C 64.38, H 6.15, Cl 6.55, N 5.18, actual C 63.73, H 6.15, Cl 6.81, N 5.07.

General directions for preparing 3,1-bridged 2-[6-(N-acetylanilino)-hexatrien-1,3,5-ylidene]-benzo[b]pyrylium and thiopyrylium perchlorates C 1586, C 1573 and C 1574, see Fig. 3:

0.005 mol of 2-methylene-7-diethylamine-benzo[b]pyrylium perchlorate of formula 1a, 1b or a 2-methylene-4,6-diphenyl-thiopyrylium perchlorate of formula 1c are dissolved in 40 ml acetic anhydride and briefly heated with 1.42 g (0.005 mol) (5-anilinopenta-2,4-dienylidene)-phenyl-ammonium chloride. The precipitate occurring after approximately one hour is sucked off, washed with ether and recrystallized from glacial acetic acid.

4: *6-Diethylamino-4-[5-(N-acetylanilino)-penta-2,4-dienylidene]-1,2,3,4-tetrahydro-<dibenzo[b;e]pyrylium> perchlorate* C 1586: Yield: 2.65 g (96%); melting point: 246-48°C. $^{-1}$H NMR (CD_3NO_2): 1.34 (t, J = 7.1 Hz, 6H), 1.84-1.88 (m, 2H), 2.08 (s, 3H), 2.67 (t, J = 5.7 Hz, 2H), 2.85 (t, J = 6 Hz, 2H), 3.72 (q, J = 7.1 Hz, 4H), 5.38 (dd, J = 11.4 Hz, J = 13.8 Hz, 1H), 6.55 (dd, J = 11.9 Hz, J = 14.3 Hz, 1H), 7.00-7.08 (m, 2H), 7.27-7.33 (m, 3H), 7.56-7.62 (m, 3H), 7.71-7.75 (m, 2H), 8.00 (d, J = 13.8 Hz, 1H), 8.12 (s, 1H). $^{-}C_{30}H_{33}ClN_2O_6$ (553.05): calculated C 65.15, H 6.01, Cl 6.41, N 5.07, actual C 63.57, H 6.08, Cl 6.14, N 4.92.

5: *3-Diethylamino-6-[5-(N-acetylanilino)-penta-2,4-dienylidene]-7,8,9,10-tetrahydro-6H<5-oxonia-cyclohepta[b]naphthalene> perchlorate* C 1573: Yield: 2.61 g (92%); melting point: 202°C. $^{-1}$H NMR (CD_3NO_2): 1.36 (t, J = 7.1 Hz, 6H), 1.78-1.82 (m, 2H), 1.90-1.94 (m, 2H), 2.01 (s, 3H), 2.76 (t, J = 6 Hz, 2H), 2.95 (t, J = 6 Hz, 2H), 3.75 (q, J = 7.1 Hz, 4H), 5.39 (dd, J = 11.3 Hz, J = 13.9 Hz, 1H), 6.57 (dd, J = 11.9 Hz, J = 14.3 Hz, 1H), 6.98-7.06 (m, 2H), 7.32-7.36 (m, 3H), 7.52-7.63 (m, 4H), 7.77 (d, J = 9.4 Hz, 1H), 7.97 (d, J = 13.8 Hz, 1H), 8.22 (s, 1H). $^{-13}$C NMR (CD_3NO_2): 12.3, 22.9, 25.2, 25.5, 25.7, 30.2, 46.8, 95.7, 114.5, 118.7, 119.1, 126.0, 127.7, 129.7, 130.1, 131.1, 131.5, 132.1, 137.8, 140.1, 142.1, 144.4, 150.8, 156.9, 159.8, 169.3, 170.3, $^{-}C_{31}H_{35}ClN_2O_6$ (567.08): calculated C 65.66, H 6.22, Cl 6.25, N 4.94, actual C 64.42, H 6.27, Cl 6.13, N 4.78.

6: *8-[5-(N-Acetylanilino)-penta-2,4-dienylidene]-2,4-diphenyl-5,6,7,8-tetrahydro-<benzo[b]thiopyrylium>percholate* C 1574: Yield: 2.37 g (79%); melting point: 216-18°C. $^{-}C_{34}H_{30}ClNO_5S$ (600.13): calculated C 68.05, H 5.04, Cl 5.91, N 2.33, S 5.34, actual C 67.34, H 5.03, Cl 5.67, N 2.24, S 5.18.

General directions for preparing 3,1-bridged 7-diethylamino-2-[3-(1-alkyl-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-propen-1-yl]-benzo[b]pyrylium perchlorates C 1592 C 1601, see Fig. 4:

In a first variant, 0.005 mol of an indole derivative 2a or 2b (Mujumdar, R. T.; Ernst, L. A.; Mujumdar, S. R.; Lewis, C. J.; Waggoner, A. S.: *Bioconjugate Chem.* 1993, 4, 105) together with 2.13 g (0.005 mol) L 107 are heated under reflux for about ten minutes in 30 ml acetic anhydride and 10 drops piperidine. After cooling, the raw product is precipitated with ethyl ether and purified by column chromatography (silica gel, methanol/acetone 1:1).

In a second variant (as indicated in Fig. 4), 2.13 g (0.005 mol) of a percholate 3b (Kanitz, A.; Hartmann, H.; Czerney, P.: J. *Prakt. Chem.* 1998, 340, 34) are used instead of L 107. It is necessary to increase the reaction time by approximately ten minutes.

7: *3-Diethylamino-6-[2-(1-n-butyl-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-ethylidenl]-7,8,9,10-tetrahydro-6H-<5-oxonia-cyclohepta[b]naphthalene> percholate* C 1592: Yield/variant A: 1.87 g (63%), yield/variant B: 1.34 g (45%); melting point: 216-18°C. - HRMS-FAB ($C_{34}H_{43}N_2O$): calculated 495.337539; actual 495.335970; D = 1.569 mmU.

8: *3-Diethylamino-6-<2-[1-(4-sulfonatobutyl)-3,3-dimethyl-5-sulfanato-1,3-dihydroindol-2-ylidene]-ethylidenl>-7,8,9,10-tetrahydro-6H-<5-oxonia-cyclohepta[b]naphthalene>potassium* C 1610: Yield: 1.25 g (36%); melting point: 216-18°C. - HRMS-FAB ($C_{34}H_{42}KN_2O_7S_2$): calculated 693.207053; actual 693.203060; D = 3.99 mmU.

9: *Absorption spectra of C 1601*: Fig. 5 shows the absorption spectrum of C 1601 in pure PBS (phosphate buffer saline) and after the addition of human serum albumin (HSA).

10: *Fluorescence spectra of C 1601*: Fig. 6 shows the emission spectra of C 1601 (excited by a 670-nm diode laser) in pure PBS and after the addition of HSA. The intensity of fluorescence was increased by a factor of 5 after the addition of HSA.

General directions for preparing 3,1-bridged 7-diethylamino-2-[3-(1-(5-carboxypentyl-3,3-dimethyl--5-sulfonato-1,3-dihydro-indol-2-ylidene)-propen-1-yl]-benzo[b]pyrylium perchlorates C 1602 and C 1591, see Fig. 7:

1.77 g (0.005 mol) of an indole derivative 2c (Mujumdar, R. T.; Ernst, L. A.; Mujumdar, S. R.; Lewis, C. J.; Waggoner, A. S.: *Bioconjugate Chem.* 1993, 4, 105) and 0.005 mol of C 1595 or L 107 are heated under reflux for about ten minutes in 40 ml of a mixture of pyridine/acetic anhydride (1/1). After cooling, the raw product is precipitated with ethyl ether and purified by column chromatography (silica gel, methanol).

11: *6-Diethylamino-4-<2-[1-(5-carboxypentyl)-3,3-dimethyl-5-sulfonato-1,3-dihydro-indol-2-yliden]-ethylidenl>-1,2,3,4-tetrahydro-<dibenzo[b;e]pyrylium>betaine C 1602*: Yield: 2.20 g (71%); melting point: >310°C. -$C_{35}H_{44}KN_2O_7S$ (657.89 * H_2O): calculated C 62.20, H 6.56, N 4.14, S 4.74, actual C 61.74, H 6.53, N 4.06, S 4.26. -HRMS-FAB ($C_{35}H_{43}N_2O_6S$): calculated 619.284184; actual 619.286390; D = -2.205 mmU.

12: *3-Diethylamino-6-<2-[1-(5-carboxypentyl)-3,3-dimethyl-5-sulfonato-1,3-dihydro-indol-2-yliden]-ethylidenl>-7,8,9,10-tetrahydro-6H-<5-oxonia-cyclohepta[b]naphthalene> betaine* C 1591: Yield: 2.15 g (68%); melting point: >340°C. -$C_{36}H_{46}KN_2O_7S$ (671.91 * H_2O): calculated C 62.68, H 6.73, N 4.06, S 4.64, actual C 62.37, H 6.61, N 4.07, S 4.34. -HRMS-FAB ($C_{36}H_{45}N_2O_6S$): calculated 633.299834; actual 633.308710; D = -8.875 mmU.

General directions for preparing NHS ester with N-hydroxysuccinimide (NHS)/N,N'-dicyclohexylcarbodiimide (DCC), see Fig. 8:

15 mg of C 1602 or C 1591, 14 mg of DCC and 4 mg of NHS are dissolved in 1 ml dry DMF and mixed with 10 µl of triethylamine. The reaction mixture is stirred for 24 hours at room temperature and subsequently filtered. After extracting the solvent, the residue is washed with ether and dried in an oil pump vacuum.

13: *C 1602 NHS ester*: The reaction runs quantitatively.

14: *C 1591 NHS ester*: The reaction runs quantitatively.

15: *Covalent labeling of human serum albumin (HSA) with C 1591 NHS ester:* C 1591 NHS ester (approximately 0.5 mg) are dissolved in 50 µl of DMF and 5 mg of HSA are dissolved in 750 µl of bicarbonate buffer (0.1 mol/l, pH = 9.0). Both solutions are gradually combined and stirred for 20 hours at room temperature. The labeled HSA is then separated from the unattached dye by gel chromatography. Sephadex G50 is used as stationary phase, phosphate buffer (22 mmol/l, pH 7.2) is used as solvent.

16: *Absorption spectra of C 1591 derivatives*: Fig. 9 shows the absorption spectrum of an activated C 1591 NHS ester and C 1591 covalently bonded to HSA. PBS (phosphate buffer saline) was used as solvent for both measurements.

17: *Fluorescence spectra of C 1591 derivatives*: Fig. 10 shows the emission spectrum of an activated C 1591 NHS ester and C 1591 covalently bonded to HSA. A 670-nm diode laser (*Spindler & Hoyer*, maximum output 3 mW) was used for excitation. PBS was used as solvent for both measurements.

18: *Fluorescence spectra of C 1591 derivatives*: Fig. 11 shows the emission spectrum of an activated C 1591 NHS ester and C 1591 covalently bonded to HSA. A 488-nm Ar-ion laser (*Ion Laser Technology*, maximum output 100 mW) was used for excitation. PBS was used as solvent for both measurements.

19: *3-Diethylamino-6-<2-[1-(3-acetoxypropyl)-3.3-dimethyl-1.3-dihydro-indol-2-yliden]-ethyliden>7.8.9.10-tetrahydro-6H-<5-oxonia-cyclohepta[b]naphthalen>perchlorate C 1594, see Fig. 12*: 1.94 g (0.005 mol) of 1-(1-acetoxypropyl)-2,3,3-trimethyl-3*H*-indolinium iodide 2 d (Brush et al., US-PS 5,808,044) and 2.13 g (0.005 mol) of L 107 are heated under reflux for approximately 20 minutes in a mixture of 20 ml pyridine and 20 ml acetic anhydride. After cooling, the intermediate stage which is still acetylated is precipitated with ether and dried under vacuum. The product is purified by preparative column chromatography (silica gel, methanol). Yield: 0.87 g (29%); melting point 155-62° C. - HRMS-FAB ($C_{35}H_{43}N_2O_3$): calculated 539.327368; actual 539.328510; D = -1.142 mmU.

20: *Preparation of C 1594 phosphoramidite, see Fig. 12*: For hydrolysis, 200 mg of C 1594 are dissolved in 10 ml methanol and stirred for two

hours while adding 50 mg of sodium carbonate, followed by filtration and the deacylated dye is precipitated by addition of ether and dried. The obtained product is dissolved in dry DMF and mixed with 0.15 ml of N,N-diisopropylamine. 40 µl of 2-cyanoethyl-N,N,-diisopropylchlorophosphoramidite are added to this solution three times over the course of an hour. The reaction is tracked by thin-film chromatography and after quantitative running of the reaction the product is used directly for labeling DNA.